Putative Temporal Variability of *Escherichia coli* Ribotypes from Yearling Steers

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ABSTRACT

***Escherichia coli* is a ubiquitous component of the intestinal microflora of warm-blooded animals, and is an indicator of fecal contamination of surface waters. Ribotype profiling of *E. coli* is one of several genotypic methods that has been developed to determine the host origin of fecal bacteria. Like most genotypic methods of source tracking, ribotyping requires a host origin database to identify environmental isolates. To determine the extent of temporal variability of ribotypes and its effect on a host origin database, *E. coli* isolates were obtained from fecal samples of two herds of Black Angus steers at a long-term experimental site at four sampling times from October 1999 to July 2000. Fecal samples were taken from six randomly chosen steers at each time. At a similarity index of 90% as calculated by unweighted pair-group method using arithmetic averages (UPGMA), 240 ribotypes were identified from 451 *E. coli* isolates. Only 20 ribotypes (8.3%), comprising 33% of the total isolates, were shared among sampling times and were considered resident ribotypes. Two of the twenty resident ribotypes appeared at three sampling times, and the remaining eighteen appeared at two. The majority of the ribotypes, therefore, were transient and unique to each sampling time and steer. Both the apparent turnover of *E. coli* ribotypes and a clonal diversity index of 0.97 (indicative of extensive ribotype variability) suggest the necessity of ribotyping a large number *E. coli* isolates per host to establish a host origin database that is independent of temporal variability, or complete enough to be effective.**

Fecal contamination of surface and ground waters can be a serious threat to public health (Geldreich, 1970). For many years, *E. coli* has been an indicator of enteric pathogens (Clesceri et al., 1998). Because *E. coli* is a component of the intestinal microflora of most warm-blooded animals, its detection alone in environmental samples is indiscriminate and it is not possible to distinguish among *E. coli* isolates from wildlife, humans, or nonpoint agricultural sources that could be associated with such pathogens as *Salmonella*, *Campylobacter*, *E. coli* O157:H7, *Shigella*, or hepatitis A virus.

In recent years, several genotypic methods have been developed for determining the host origin of fecal bacteria in contaminated waters, a technique referred to as *microbial source tracking*. These methods include ribotyping (Parveen et al., 1999; Hill et al., 2001; Carson et al., 2001), pulse field gel electrophoresis (PFGE) (Kariuki et al., 1999), and various polymerase chain reaction (PCR)–based methods (Bernhard and Field, 2000a,b; Dombek et al., 2000; Farnleitner et al., 2000). All of these methods are based on host-specific genetic markers (Gordon, 2001).

Ribotyping takes advantage of the conserved nature of the ribosome and its genes (Grimont and Grimont, 1986), and has been considered an effective genotypic method because it has excellent reproducibility, good discriminatory power, ease of interpretation, ease of performance (Farber, 1996), and may be automated (Hartel et al., 1999; De Cesare et al., 2001). Ribotyping has identified various host origins of *E. coli* in a contaminated creek (Farag et al., 2001), distinguished between human and nonhuman sources in an estuary in Florida (Parveen et al., 1999), and determined the source of fecal coliform contamination in household wells (Hill et al., 2001). Identifying the host origin of *E. coli* could solve two environmental problems: (i) it could determine the health risk associated with *E. coli* by distinguishing between human and nonhuman sources in environmental water samples, and (ii) it could identify a point or nonpoint source to facilitate the reduction or elimination of the polluting source, and thus be useful regarding total maximum daily load (TMDL) regulations. To match animal hosts and environmental isolates, ribotyping and most other genotypic methods require a host origin database.

Early work on the population genetics of *E. coli* established that *E. coli* was clonal: it depended on asexual reproduction (vertical gene transfer) and low rates of genetic recombination (lateral gene transfer) (Milkman, 1973; Selander and Levin, 1980). The estimated number of clones of *E. coli* in a natural population associated with a particular host species could range between 100 and 1000 (Selander et al., 1987). For methods of microbial source tracking to be effective, the clonal population of *E. coli* should be stable through time (Gordon, 2001). Using multilocus enzyme electrophoresis, Caugant et al. (1981) reported extensive temporal variation of electrophoretic types (ET) of *E. coli* from a single human; of 53 ETs identified, only three appeared to persist over time and were considered resident types. Similar results were observed in an *E. coli* population from 447 feral house mice in Australia (Gordon 1997). Temporal variability of *E. coli* O157:H7 genotypes for herds of dairy cows has also been observed (Faith et al., 1996).

The temporal variability of any host origin database, therefore, needs to be addressed. Variability of ribotypes in a particular host over time could have significant logistical ramifications for ribotyping as a microbial source tracking method because it may require continuous updating of the host origin database. The objective of this study was to determine the extent of temporal variability of *E. coli* ribotypes obtained from yearling steers over a one-year duration.

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Abbreviations: ATCC, American Type Culture Collection; ET, electrophoretic type.

MATERIALS AND METHODS

Sampling Scheme

Yearling Angus steers (*Bos taurus*) (Herd 1) were calved at the J. Phil Campbell, Sr., Natural Resource Conservation Center (Watkinsville, GA), and after being weaned in October 1998, were transported to the long-term experimental site. The second group of yearling Angus steers (Herd 2) also were calved at the center and were transported to the experimental site after being weaned in October 1999. The long-term experimental site is a 15-ha upland (33°22′ N, 83°24′ W) in the Greenbrier subwatershed of the Oconee River watershed near Farmington, GA (Franzluebbers et al., 2001). The mean annual temperature is 16.5°C, rainfall is 1250 mm, and potential evaporation is 1560 mm. The site is comprised of 18 experimental paddocks, 0.69 ± 0.03 ha each. The number of steers per paddock was determined by the mass of forage above ground level. Spatial design of the paddocks minimizes runoff contamination and handling of animals through a central roadway. Each paddock contained a 3- by 4-m shade, mineral feeder, and a water trough placed in a line 15 m long near the top of the landscape. Paddocks were fertilized either with inorganic fertilizer or poultry litter. The forage composition was coastal bermudagrass [*Cynadon dactylon* (L.) Pers.] interseeded with 'Georgia 5' tall fescue (*Festuca arundinacea* Schreb.). At each sampling, six different Angus steers were randomly chosen. No steer was sampled more than once. The first sampling occurred in October 1999 at the end of the grazing season, and just before the steers were sent to drylot for finishing. The second sampling (Day 1 for Herd 2) was in March 2000, with subsequent samples obtained in May 2000 (71 d) and July 2000 (129 d). Further sampling was not possible because drought conditions necessitated the removal of the steers off the experimental site. The six randomly sampled steers grazed on paddocks that had been fertilized with poultry litter or inorganic fertilizer, and the paddock fertilization treatments were not considered as a factor in the experimental design. Thus, in this experimental scheme, the experimental pasture was the same for each herd; herds were from the same gene pool and were differentiated by chronology.

Selection and Identification of *Escherichia coli* Isolates

At the time of each sampling, fecal samples were obtained by digital retrieval from the rectum of each steer. Sterile latex gloves were worn for each steer. The fecal samples were placed into sterile Nasco (Fort Atkinson, WI) Whirl-Pak bags, placed on ice, and transported to the lab where they were stored at 4°C. All samples were processed within 24 h. Fresh feces (approximately 10 g) from each steer were suspended in 90 mL of sterile 0.1% peptone broth contained in a 160-mL milk dilution bottle, shaken to suspend solids, and aseptically streaked on 5-cm Petri dishes containing mTEC medium (Difco Laboratories, Sparks, MD) for isolating thermotolerant *E. coli*. Duplicate plates were incubated submerged in a water bath at 44.5 ± 0.2°C for 24 h according to standard methods (Clesceri et al., 1998). Preincubation of the inoculated mTEC plates at 35°C was not performed because we considered the fraction of stressed *E. coli* from fresh feces to be insubstantial. Yellow colonies on the medium were randomly selected, streaked onto tryptic soy agar (Difco), and incubated at 35°C for 24 h. The streaking was repeated twice to ensure the purity of each isolate. Each isolate was inoculated into a 24-multiwell tissue culture plate containing separate 1-mL slants of Simmons citrate and urea agar (both Difco). Three bacterial species from the American Type Culture Collection (ATCC; Manassas, VA) were used as controls: *E. coli* ATCC #11775 (citrate negative, urea hydrolysis negative), *Klebsiella pneumoniae* ATCC #13883 (citrate positive, urea hydrolysis positive), and *Enterobacter aerogenes* ATCC #13048 (citrate positive, urea hydrolysis negative). Isolates that were both citrate-negative on Simmons citrate agar and urea hydrolysis negative on urea agar were subjected to an oxidase test (MacFaddin, 1976). Isolates that were oxidase negative were considered *E. coli* and kept for long-term storage. Thus, a loopful of each isolate (approximately 40 mg) was removed from a tryptic soy agar plate and placed in a cryovial containing 900 μL of cryoprotectant mixture consisting of 700 μL of saline–phosphate (NaCl, 8.5 g L^{-1}; K_2HPO_4, 0.65 g L^{-1}; KH_2PO_4, 0.35 g L^{-1}; pH 7.0), 100 μL of glycerol, and 100 dimethyl sulfoxide. Isolates were stored at −80°C.

DNA Extraction and Quantification

Isolates of *E. coli* were streaked on tryptic soy agar and incubated at 35°C for 24 h. A single colony was inoculated into 10 mL of Luria–Bertani broth (pH 7.5; Sambrook et al., 1989) contained in a 16- by 150-mm test tube and secured flat on a rotating shaker at 75 rpm at 35°C. After 18 h, a 2.0-mL sample was removed and the DNA extracted with a commercial kit (Qiagen DNeasy; Qiagen, Valencia, CA). A portion of the DNA was mixed with Hoechst Dye #33258 (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's directions, and the DNA was quantified with a fluorometer (DynaQuant DQ200; Amersham Pharmacia Biotech). DNA from *E. coli* strain B (Sigma Chemical Co., St. Louis, MO) was the standard.

Ribotyping of *Escherichia coli* Isolates

A standard ribotyping protocol was followed, similar to that of Parveen et al. (1999). Briefly, two 1-μg samples of DNA from each isolate were each separately digested overnight with the restriction enzymes *Eco*RI and *Pvu*II. The digested DNA was stained and was loaded into a 1% agarose gel. The gel was electrophoresed at 58 V for 3 h with a horizontal gel system. Digoxigenin (DIG)-labeled Marker III (Roche Molecular Biochemicals, Indianapolis, IN) was the molecular weight marker and occupied every fifth lane of the gel. Control lanes contained no DNA (control) and DNA from *E. coli* ATCC #11775. DNA was transferred by Southern blotting to a nylon membrane with a vacuum blotting system and the DNA on the membrane was crosslinked with UV light. Following a 2-h prehybridization at 42°C, the membrane was hybridized at the same temperature overnight to DIG-labeled cDNA from *E. coli* total ribosomal RNA (Hartel et al., 2002). Membranes were prepared for chemiluminescence by a series of washing steps before a chemiluminescent substrate for alkaline phosphatase was added. Membranes were placed in a FluorChem 8000 imager (Alpha Innotech, San Leandro, CA) and images saved as TIFF files. TIFF files were imported into GelCompar II (Applied Maths, Kortrijk, Belgium) for analysis. Typically, gels showed 9 to 11 bands for *Eco*RI and 11 to 13 bands for *Pvu*II digestion, and this was considered sufficient for good discrimination among ribotypes. DNA fragments < 1375 base pairs were ignored because they were often indistinct. Lanes were normalized within the gel with the molecular weight marker and variations among the gels were assessed with the *E. coli* ATCC #11775 strain. Optimization (maximum percentage shift allowed between two different patterns for the patterns to still be considered a match) and tolerance (maximum percentage shift allowed between two bands on different patterns for the bands to still be considered a match) were each set at

1.00%. The normalized banding patterns for both enzymes were stacked with *Eco*RI on the top and *Pvu*II on the bottom to create one combined ribotype pattern for each isolate. Similarity indices, the percentage similarity between each banding pattern, were determined with Dice's coincidence index (Dice, 1945) and the distance between clusters calculated with the unweighted pair-group method with arithmetic averages (UPGMA). The banding pattern of the control *E. coli* ATCC #11775 strain varied from gel to gel. The variations in banding patterns of the control *E. coli* ATCC #11775 strain were attributed not to genetic changes or changes in the purified DNA, but to slight variations in DNA concentration and irregularities in the casting of agarose gels. Based on this variability, the banding patterns of all other isolates had to have a similarity index of 90.0% to be considered the same ribotype.

Data Analysis

Statistical analyses of the isolate to ribotype ratios among the sampling times for Herd 2 (no data on individual steers from Herd 1 were recorded) were performed with Minitab statistical software (Minitab, 2002), and the probability value of at least 0.05 was used to determine significant differences. Clonal diversity is defined as $d = n(1 - x_i^2)/n - 1$, where x_i is the relative abundance of the ith ribotype and n is the total number of isolates characterized (Whittam, 1989). A clonal diversity index of 0 indicates the presence of a single ribotype, and an index of 1 indicates 100% diversity, or each isolate represents a ribotype.

RESULTS AND DISCUSSION

A total of 451 *E. coli* isolates was obtained from four sampling times and two herds of yearling steers (Table 1). Based on a 90% similarity index (dendrogram not shown), 240 ribotypes were identified. The correlation between the number of isolates and number of ribotypes was 0.57 and not significant ($P = 0.25$). The clonal diversity remained high (0.93–0.99) throughout the sampling period (October 1999–July 2000). The mean isolate to ribotype ratio for Herd 2 did not change significantly between Days 1 and 71, but did change significantly between Days 1 and 129; thus, the isolate to ribotype ratio appears to have potential to vary significantly over time. The ribotype diversity within the cattle tested at any sampling time appeared to be as extensive as the ribotype diversity between a collection of *E. coli* isolates from diverse hosts. The overall isolate to ribotype ratio of 1.8:1 (as well as the isolate to ribotype ratios for each sampling time) was similar to the 1.7:1 isolate to ribotype ratio (179 *E. coli* isolates yielded 102 ribotypes) observed by Parveen et al. (1999) for *E. coli* isolates from both human and a variety of nonhuman sources.

The number of ribotypes observed was dependent on the cutoff of the similarity index. The relationship between the similarity cutoff and number of ribotypes is proportional; a low similarity index will yield fewer ribotypes and a greater percentage of sharing and a high similarity index will yield more ribotypes and a lesser percentage of sharing. In our research, a 90% similarity index yielded 240 ribotypes. Dombek et al. (2000) used BOX A1R primers and rep-PCR (polymerase chain reaction) to analyze 21 *E. coli* isolates from 12 cows at a 75% similarity index. This resulted in four distinct clusters with 67% of the isolates subsumed under one cluster. If our *E. coli* isolates were analyzed at a 75% similarity, then only 36 ribotypes would be identified and 62.5% of the isolates would be considered resident. We used a 90% similarity index because this was the cutoff required for all *E. coli* ATCC #11775 intergel controls to be considered the same ribotype. This similarity index was the same as for the *E. coli* isolates that Hartel et al. (2002) ribotyped. If the estimated range of clones in a natural population of *E. coli* is 100 to 1000 (Selander et al., 1987), then both the number of bovine genotypes that Dombek et al. (2000) observed and the number of ribotypes that we observed at a 75% similarity index appear to underestimate the clonal diversity of the populations tested.

Table 1. Number of isolates, number of ribotypes, isolate to ribotype ratio, and clonal diversity of *Escherichia coli* obtained from the feces of six randomly selected steers. Both herds were on the same paddocks at an experimental pasture near Farmington, GA. Herd 1 was sampled in October 1999 and Herd 2 sampling began in March 2000.

Characteristic	Herd 1	Herd 2 (Day 1)	Herd 2 (Day 71)	Herd 2 (Day 129)	Total
Number of isolates	65	99	140	147	451
Number of ribotypes	24	81	85	72	240
Isolates to ribotype ratio†	2.7:1	1.1:1a	1.6:1ab	1.7:1b	1.8:1
Clonal diversity	0.93	0.99	0.99	0.98	0.97

† Mean isolate to ribotype ratios of the six steers per sampling time of Herd 2 that are followed by different letters are significantly different at $P < 0.05$.

Of the 240 ribotypes, 183 were unique (i.e., observed only at one sampling time and only from one steer). The remaining 57 ribotypes shared more than one isolate (Fig. 1). Twenty-four different ribotypes shared two isolates each, six different ribotypes shared three isolates each, and a few single ribotypes shared 9, 10, 12, 15 and 20 isolates each. The frequency of ribotypes sharing the same number of isolates decreased as the number of isolates shared by a ribotype increased. Caugant et al. (1981) observed a similar frequency pattern in their distribution of ETs.

Following the nomenclature of Caugant et al. (1981), a ribotype was considered transient if it was observed at only one sampling time and resident if observed at more than one sampling time. In addition to the 183 unique ribotypes, 37 shared ribotypes were observed at only one sampling time. Therefore, of 240 ribotypes, the vast majority (220 or 91.7%) were transient and only 20 ribotypes were resident (8.4%). Of these remaining resident ribotypes, 18 (90.0%) were observed at two sampling times and two (Ribotypes 18 and 80) were observed at three sampling times (Table 2). No ribotypes were observed at all four sampling times. Thirteen of the 30 resident ribotypes were shared between steers per sampling time; the remaining resident ribotypes were observed in one steer per sampling time. Ribotype 18 was the only resident ribotype that was observed in more than one steer of Herd 2 as well as at two sampling times. Five of the resident ribotypes were associated with Herd 1 and 15 with Herd 2. Of the five resident ribotypes from steers of Herd 1, none was observed for the initial sampling of Herd 2, two were shared with

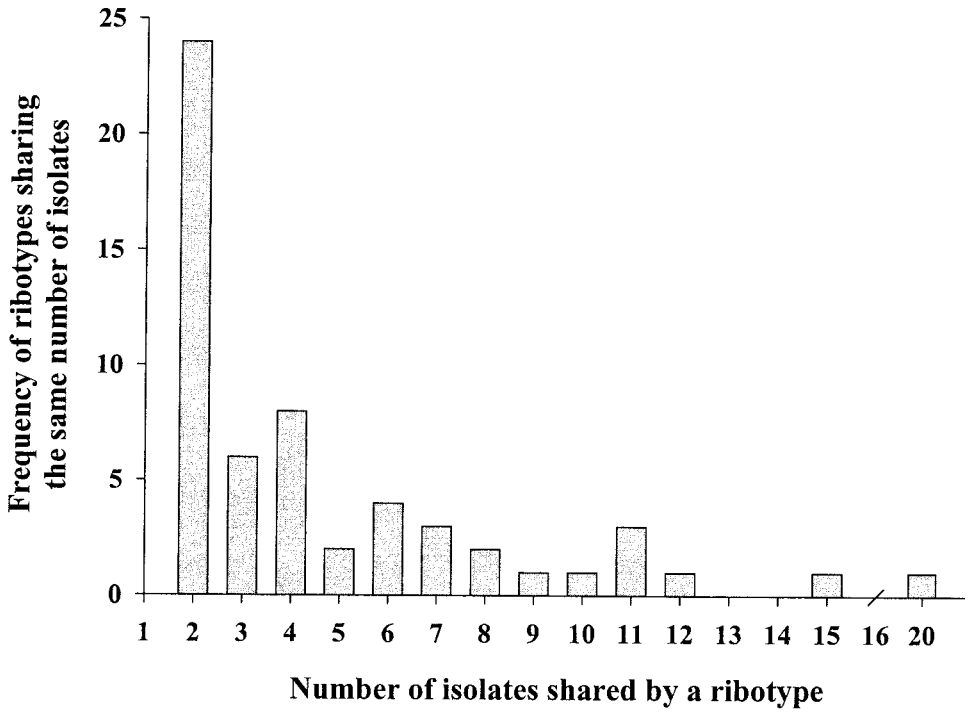

Fig. 1. The frequency of ribotypes sharing two or more isolates (*y* axis) plotted against the number of isolates that are shared by a ribotype (*x* axis).

isolates from the Day 71 sampling, and four were shared with isolates from the Day 129 sampling. The percent of resident ribotypes for each of the four sampling times was 25.0, 13.4, 12.9, and 19.4, respectively. The ratio of transient ribotype to resident ribotype was 22.6:1.

Two possible explanations for these data are apparent. The first explanation parallels the studies of Caugant et al. (1981) and Gordon (1997). Caugant et al. (1981) observed that 50 of 53 ETs of *E. coli* appeared only once over their 11-mo sampling period, and of the three ETs that did persist, none appeared at every sampling time. Therefore, only these three persistent ETs were considered resident clones of the host. Gordon (1997) observed both resident and transient clones in feral mouse populations. In our study the majority of the *E. coli* isolates had ribotypes unique to the time of sampling and to the steer sampled. Therefore, most ribotypes were transient. If resident ribotypes were defined as ribotypes shared between herds, and among sampling dates, then 8.3% of the ribotypes were considered residents and the isolates found in them (32.6%) were persistent resident clones. Because only a small percentage of ribotypes were resident the turnover or flux of ribotypes appeared to be substantial. Others have also noted temporal changes in *E. coli* subspecies. Using pulse field gel electrophoresis (PFGE) and restriction endonuclease digestion profiles (REDP), Faith et al. (1996) observed that *E. coli* O157:H7 isolates in a herd of cattle changed over time, and different REDPs were observed among cows within a herd. These studies, as well as ours, suggest that a large number of transient clones exist, or conversely, that few resident ribotypes exist.

The second explanation is that a large number (e.g., 1000 as Selander et al. [1987] suggested) of *E. coli* subspecies per host species exists at all times and a small amount of sampling (<150 isolates at each sampling period) gives the appearance as if temporal variability existed. Samadpour and Chechowitz (1995) suggest the scenario that all ribotypes are resident ribotypes and transient ribotypes do not exist. Knowing that a large number of ribotypes existed before the experiments were conducted was not possible. Whether the clones were or were not in a constant state of flux, our data suggest that a large sampling is needed for a host origin

Table 2. Distribution of resident ribotypes and isolates among sampling times. To be a resident, a ribotype must be observed at two or more sampling times.

	Number of isolates			
	Sampling			
Ribotype	Herd 1	Herd 2 (Day 1)	Herd 2 (Day 71)	Herd 2 (Day 129)
14	0	1	0	1
18	0	3	5	12
32	0	0	4	7
36	1	0	0	2
38	0	1	1	0
40	0	2	0	4
47	4	0	4	0
51	11	0	0	1
57	5	0	0	6
71	0	1	1	0
73	0	1	0	5
75	0	0	3	5
80	8	0	4	3
84	0	1	0	1
89	9	0	0	1
101	0	5	0	2
109	0	4	5	0
138	0	5	1	0
152	0	2	2	0
233	0	0	2	1
Total resident isolates	30	26	32	51
% of shared ribotypes	25.0	13.4	12.9	19.4

database. If the effective number of ribotypes in an *E. coli* population is >400 like that for alleles (Milkman, 1973), and the isolate to ribotype ratio is approximately 2:1, then between 900 and 1000 isolates would have to be ribotyped per host to identify all or nearly all the ribotypes of a host population, whether transient or resident. If a host population of *E. coli* was comprised of as many as 1000 clones as suggested by Selander et al. (1987) and each clone represented a ribotype, then ≥2000 isolates per host species may be necessary to ribotype.

CONCLUSIONS

Based on a 90% similarity index, ribotyping *E. coli* isolates from yearling steers showed a putative temporal variability of ribotypes. Given that for any of the sampling times, ≤21% of the ribotypes were resident ribotypes, and the clonal diversity indices ranged from 0.93 to 0.99, a small sample size of *E. coli* ribotypes from a particular host may be either a snapshot of a clonal population in constant flux or a mere fraction of a much larger resident population. Increasing the sample size (e.g., to 1000) per individual host may increase the number of ribotypes observed, but may not alter the apparent 20:1 transient ribotype to resident ribotype ratio observed in this study. Our results suggest that for a host origin database to be effective, a large number (≥900) of *E. coli* isolates may need to be ribotyped for each host, and the database may need to be established as contemporaneously as possible to the collection of environmental samples.

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